Supplementary Information

Dynamically induced spatial segregation in multispecies bacterial bioconvection

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Figs. S1 to S15
Table S1

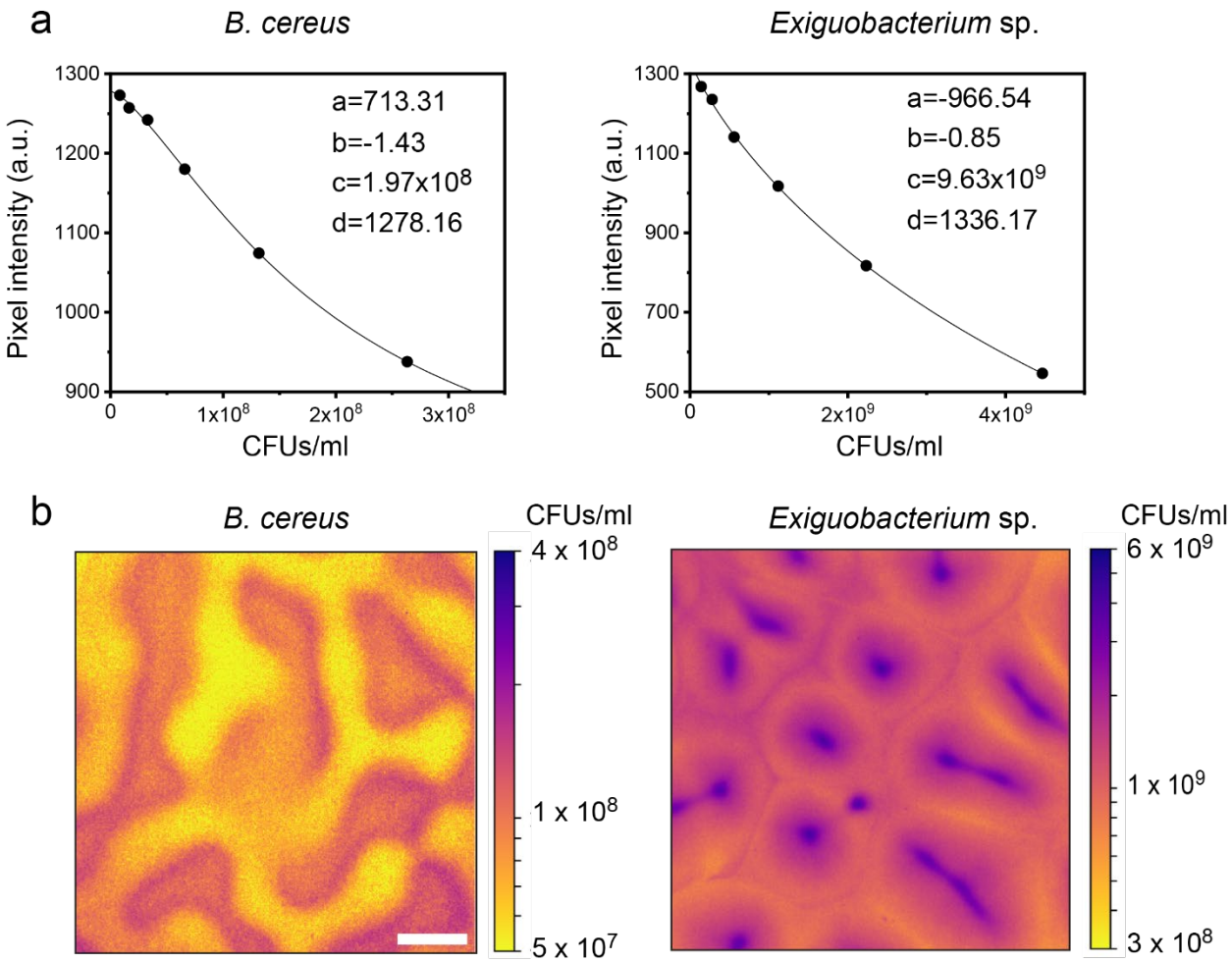


Fig. S1. Bacterial density across bioconvective patterns. a Calibration of pixel transmitted light intensity in terms of bacterial concentration, for patterns obtained with *B. cereus* and *Exiguobacterium* sp. Full circles represent measurements of mean intensity values and the curve is a fit to the data with the Rodbard function[1]**:**

$$f(x) = c\left(\frac{x-a}{d-x}\right)^{1/b}$$

The value of the parameters of the fit a, b, c and d are given for the indicated species in the corresponding panels. **b** Heat maps of transmitted light intensity calibrated in terms of colony forming units (CFUs) for bioconvection patterns in *B. cereus* and *Exiguobacterium*. Images were taken 1.5 h after initiation of bioconvection. Color-coded bars denote concentrations in CFUs·mL^{-1}. The difference in concentration between the plume region and the edge of the convection cells is more pronounced for *Exiguobacterium* sp. than for *B. cereus*. Scale bar = 1 mm.

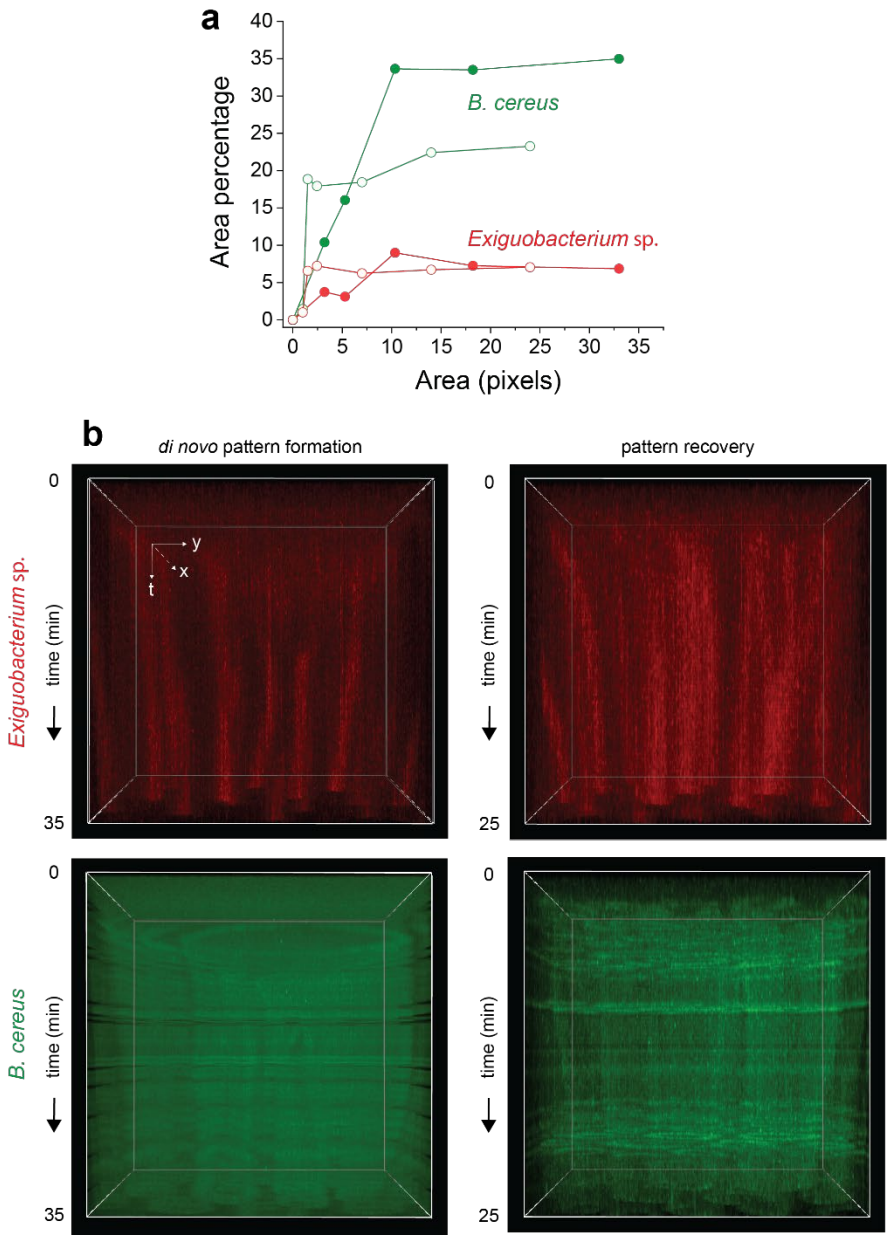


Fig. S2. Dynamics of spatial segregation and recovery after mixing in a two-species suspension. Binary suspension of *Exiguobacterium* sp. (red) and *B. cereus* (green) undergoing both *de novo* DISS and recovery following suspension mixing to homogeneity. **a** Relative area percentage covered by bioconvective down-flowing currents of both species for *de novo* DISS (full circles, corresponding to the bottom panels of Fig. 2) and recovery (empty circles). For more details see section *Image analysis of down-flowing currents of fluorescently labeled species* above. **b** Space-time diagrams (x,y,t) of the indicated bioconvective flows corresponding to Movie S3 (left panels), and rapid pattern recovery following mixing of the binary suspension to homogeneity (right panels). The (x,y) coordinates correspond to patterns viewed from the top of the well as in the movie.

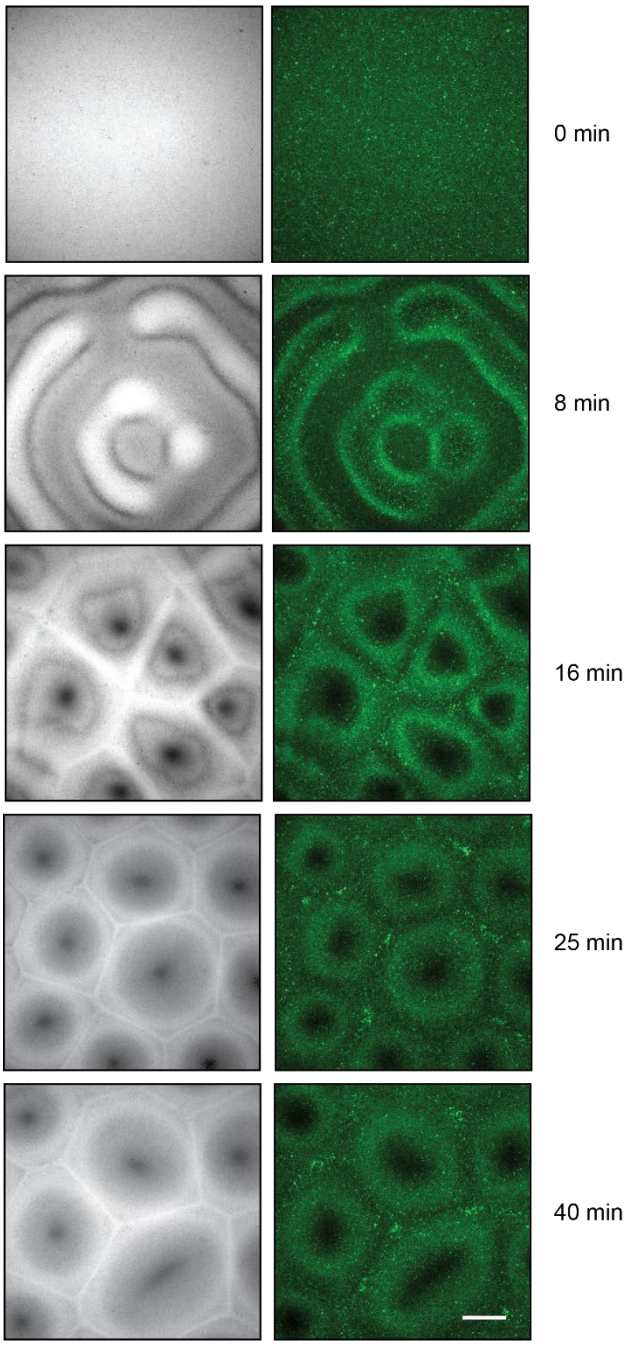


Fig. S3. Bioconvection and DISS in a mixed suspension of *Exiguobacterium* sp. and *B. cereus*. Suspensions of the constituent species were mixed in equal volume ratios, with *B. cereus* carrying the plasmid pNW-sfGFP (SPS6) that constitutively expresses the green fluorescent protein. Bright-field images are on the left with the corresponding green channel image on the right. As observed using the fluorescent dyes, the green channel image shows that the *B. cereus* plume (green regions) separates from the *Exiguobacterium* sp. plume (non-labeled region in the center). Scale bar = 1 mm. The images are representative of two independent experiments.

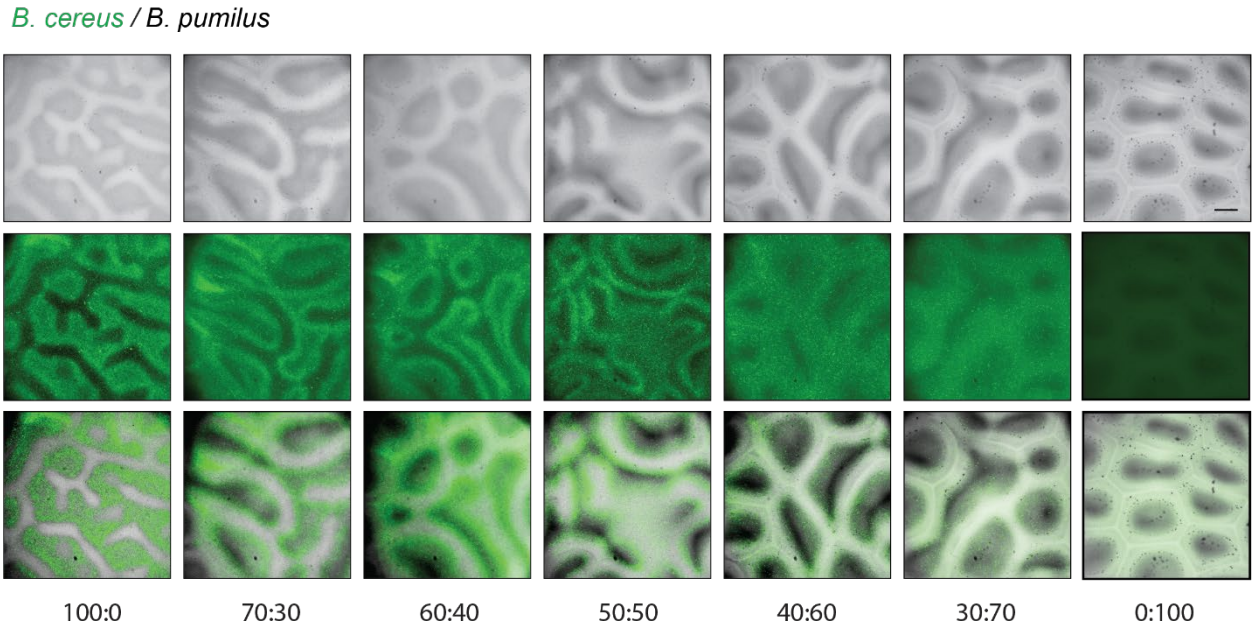


Fig. S4. DISS bioconvective patterns as a function of the relative concentration of constituent species in binary suspensions. Bioconvective patterns in binary suspensions of *B. pumilus* and *B. cereus*, the latter carrying the plasmid pNW-sfGFP (SPS6) that constitutively expresses the super folder green fluorescent protein (see Methods), at the indicated relative volume ratios of the two constituent species. Top panels show images of patterns taken by transmitted light microscopy, recorded 30 min following mixing. Middle panels: green channel with GFP signal showing the same fields of view. Bottom panels show overlays of transmitted light and green fluorescence channels (see Methods). *B. pumilus* flows down at the center of nested convective cells, surrounded by down currents of *B. cereus*, which is resistant to antagonism by *B. pumilus*[4]. Scale bar = 1 mm. The images are representative of three independent experiments.

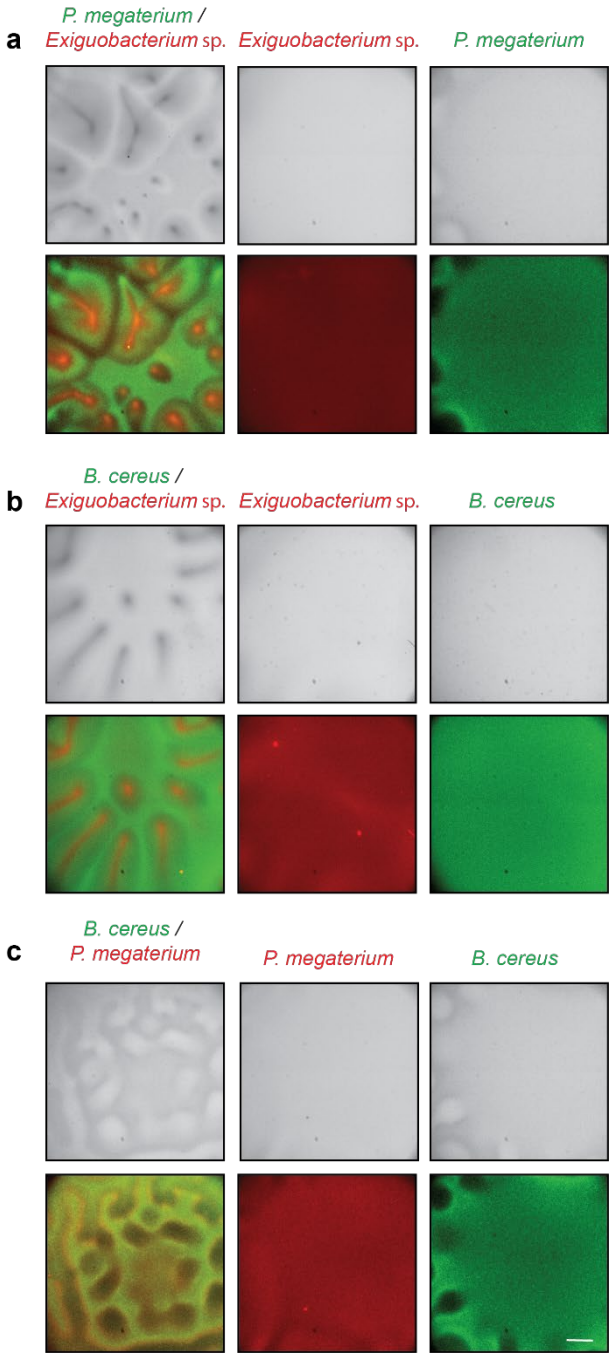
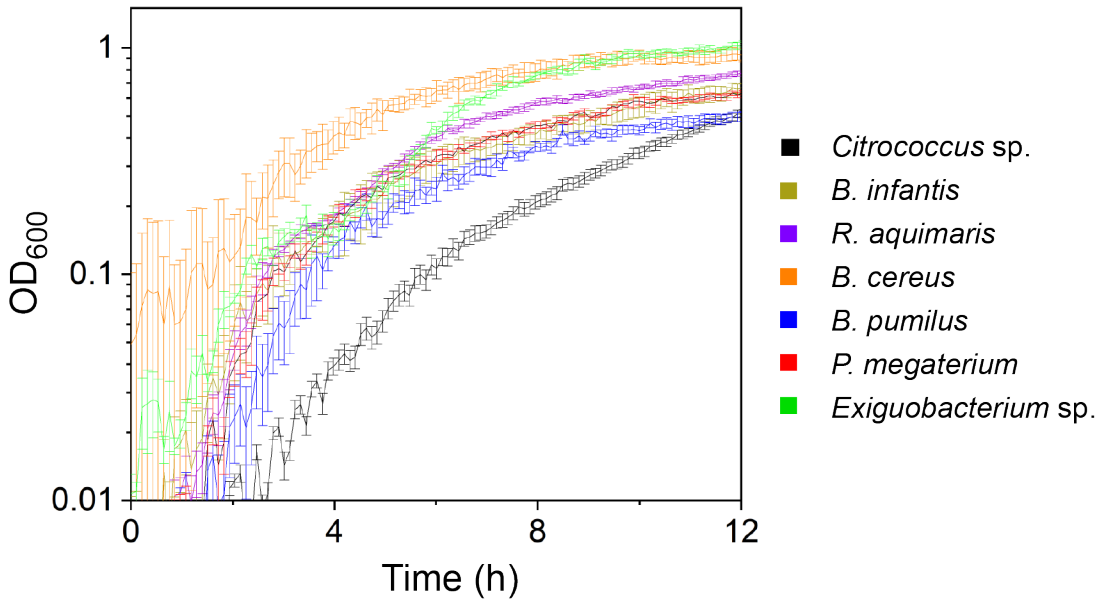


Fig. S5. The onset of bioconvection is determined by the total bacterial concentration in a multispecies suspension. Upper and bottom panels correspond to bright field and fluorescent channels, respectively. Left panels correspond to the indicated binary suspension 30 min following mixing; central and right panels correspond to each species diluted 50:50 with marine medium to obtain the same species concentration as in the mixture. **a** Suspension of *Exiguobacterium* sp. and *P. megaterium* **b** Suspension of *Exiguobacterium* sp. and *B. cereus*. **c** Suspension of *B. cereus* and *P. megaterium*. Scale bar = 1 mm. The images are representative of three independent experiments.

a



b

Species	Doubling time in post-exponential phase (h)
Citrococcus sp.	1.63±0.06
B. infantis	1.76±0.07
R. aquimaris	2.32±0.09
B. cereus	1.82±0.09
B. pumilus	1.23±0.09
P. megaterium	1.55±0.05
Exiguobacterium sp.	1.57±0.07

Fig. S6. Doubling time of species. a Growth curves of the indicated species. Four overnight cultures for each species were adjusted to an OD_{600} of 0.01 and inoculated in a 96-well plate with mineral oil on top to prevent evaporation. The plate was incubated at 28 °C with 6 minutes intervals between measurements in a plate reader. Error bars represent standard deviations of the mean. Four independent experiments were measured in parallel for each species. **b** Species doubling times determined from the region in the growth curves corresponding to the post-exponential phase, in which motility is maximal, and in which bioconvection takes place[2,3]. Errors represent uncertainties of power law fits to the data (see Methods).

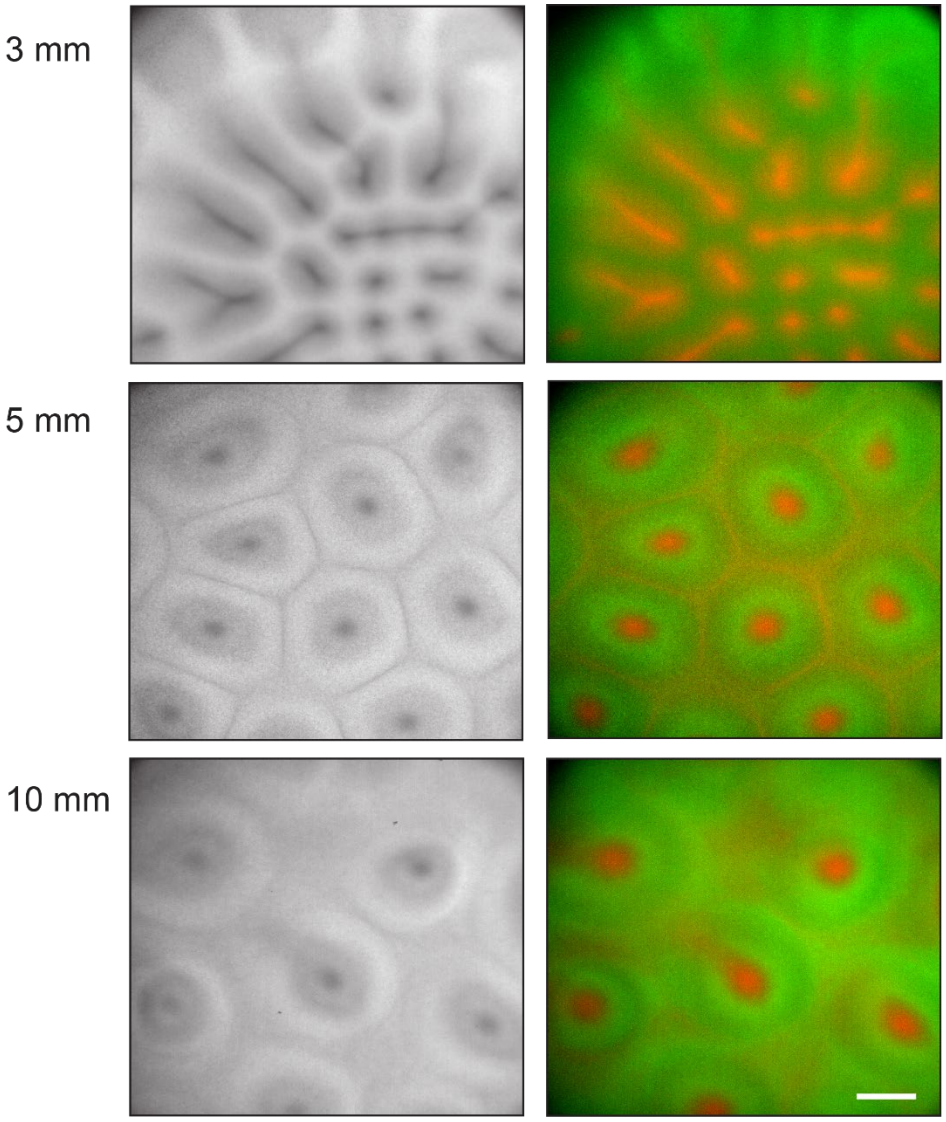


Fig. S7. Segregation in bacterial suspensions of different heights in a well. Bioconvection and segregation in a 50:50 mixed bacterial suspension of *Exiguobacterium* sp. (red) and *B. cereus* (green), for the given suspension heights. The left column corresponds to the bright field channel, whereas the right column is an overlay of fluorescence channels. Scale bar = 1 mm. The images are representative of two independent experiments.

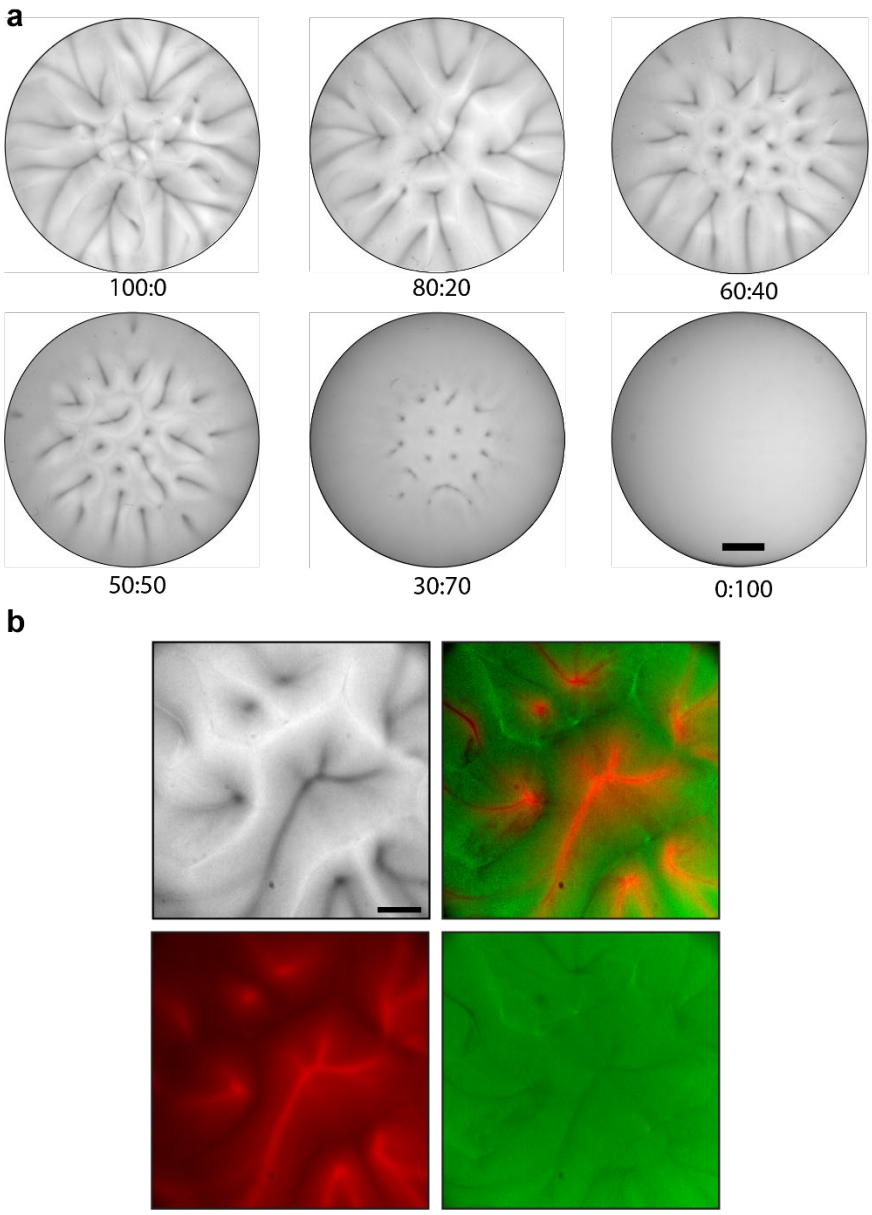


Fig. S8. Bioconvection pattern in a motile/sessile binary species suspension. a *Exiguobacterium* sp./*Citrococcus* sp. mixtures at different volume ratios show that increasing the concentration of the non-motile *Citrococcus* sp. affects the bioconvection pattern of *Exiguobacterium* sp. The numbers below each panel represent the proportion of *Exiguobacterium* to *Citrococcus.* As the concentration of *Citrococcus* increases, bioconvection near the rim gradually disappears, and the activity becomes confined primarily to the central region of the well. Scale bar = 2 mm. The images are representative of three independent experiments. **b** Bioconvection in the central region of a *Exiguobacterium* sp./*Citrococcus* sp. mixed suspension (30:70), with both species labeled: *Exiguobacterium* sp. (red) and *Citrococcus* sp. (green). See also Movie S5. Scale bar = 1 mm. The images are representative of two independent experiments.

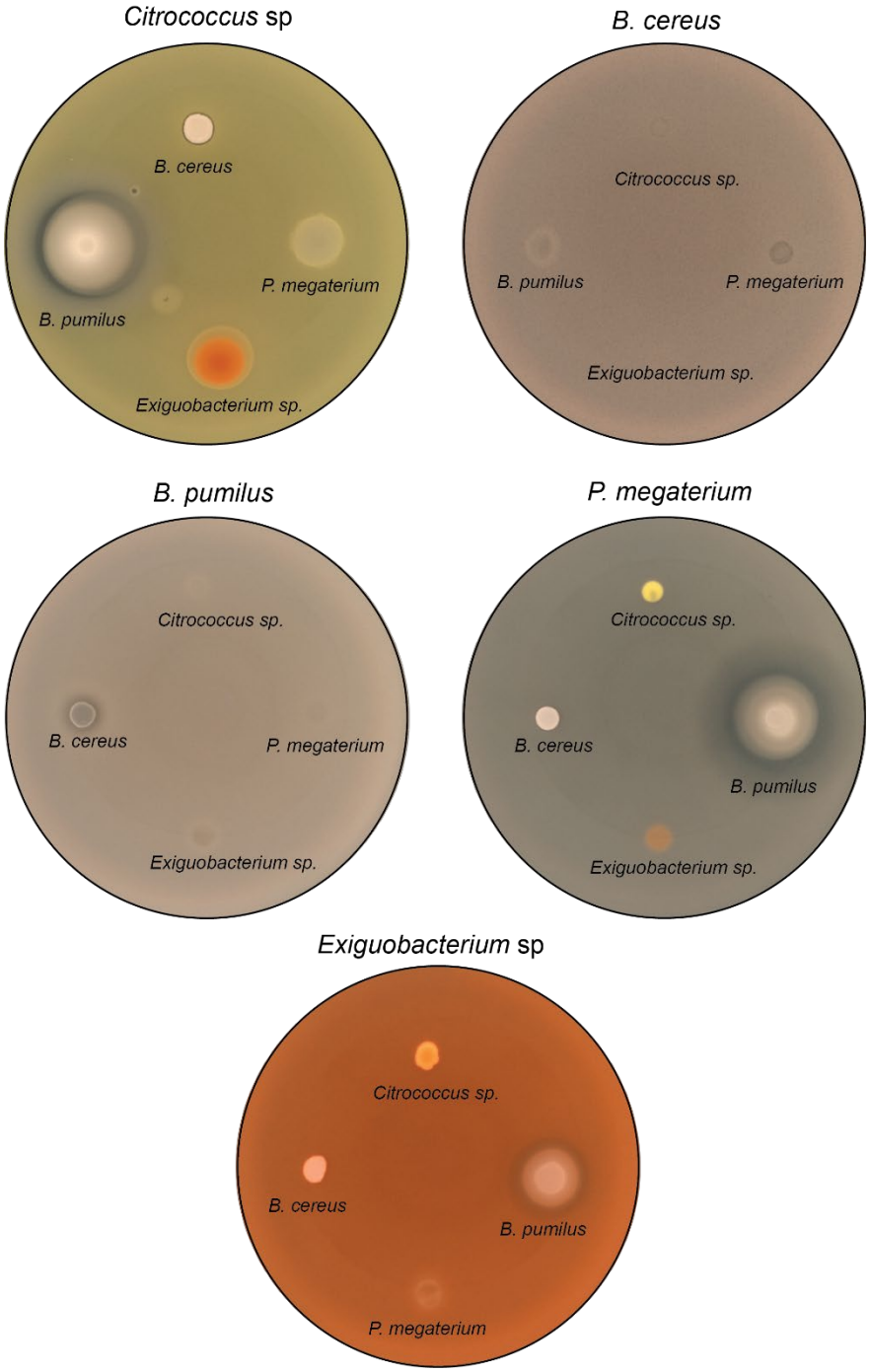


Fig. S9. Pairwise interactions as measured by spot-lawn assays. Spot-lawn antagonism assays were performed to determine the antagonism interactions among the indicated species. The presence of an inhibition halo around a spot indicates its antagonism toward the species forming the lawn, indicated at the top of each plate. The images are representative of three independent experiments.

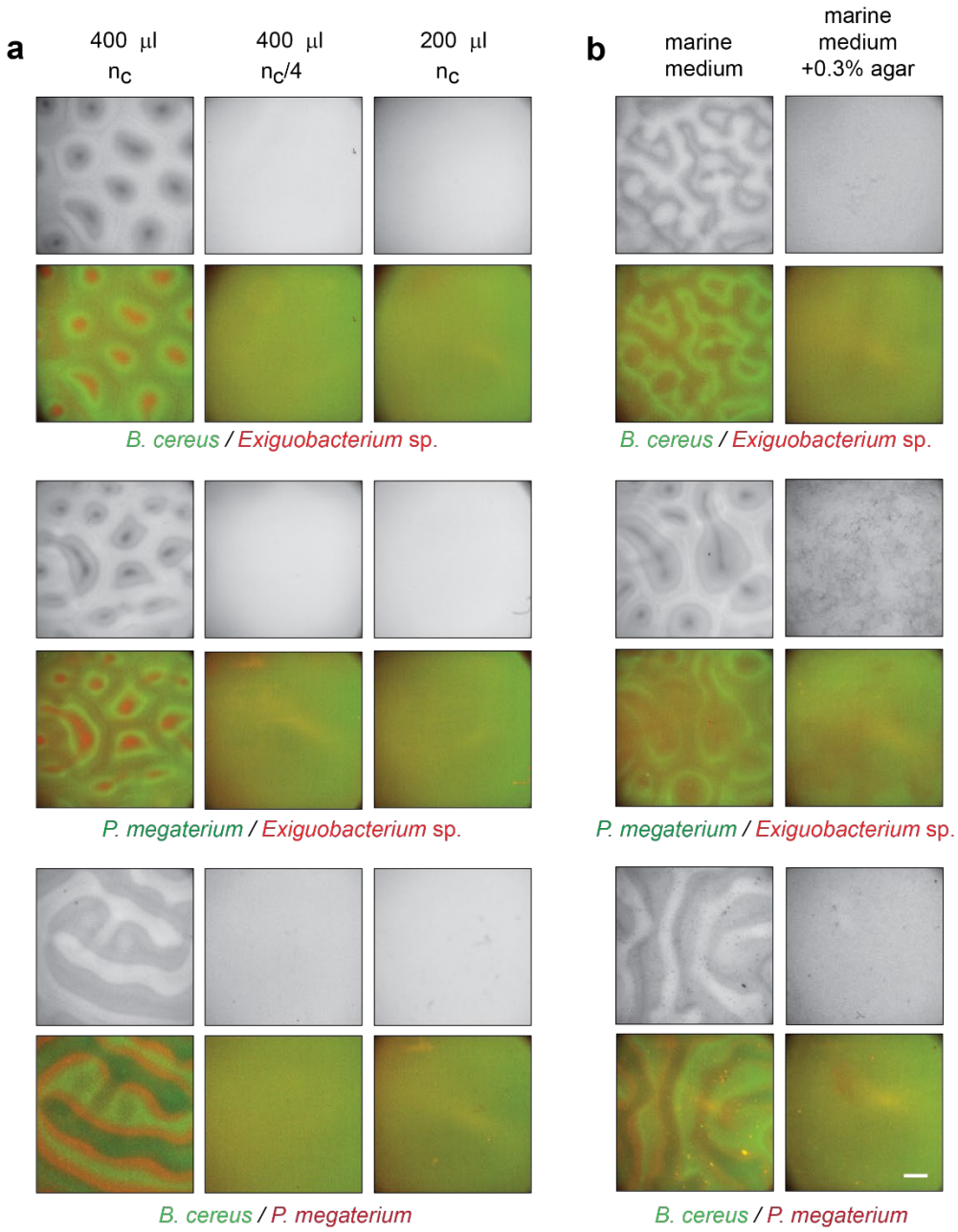


Fig. S10. Segregation observed above the onset of bioconvection. a Left panels: Transmitted light (top) and fluorescence (bottom) imaging of the indicated binary suspensions in marine medium and bacterial concentrations (note: $n_c \sim 10^9$ cells/mL) in a 400 µl suspension. Middle panels: same suspensions, but at a fourth of the bacterial concentration ($n_c/4$). Right panels: same as in left panels but in suspensions of 200 µl. Conditions in the middle and right panels are below those needed to attain the bioconvection onset. For each binary suspension, the images were recorded at the same time (30-60 min). **b** Transmitted light and fluorescence imaging of the same suspensions at a volume 400 µl, under the same conditions as in **a**, in either marine medium, or marine medium with 0.3% agar. Scale bar = 1 mm. The images are representative of three independent experiments.

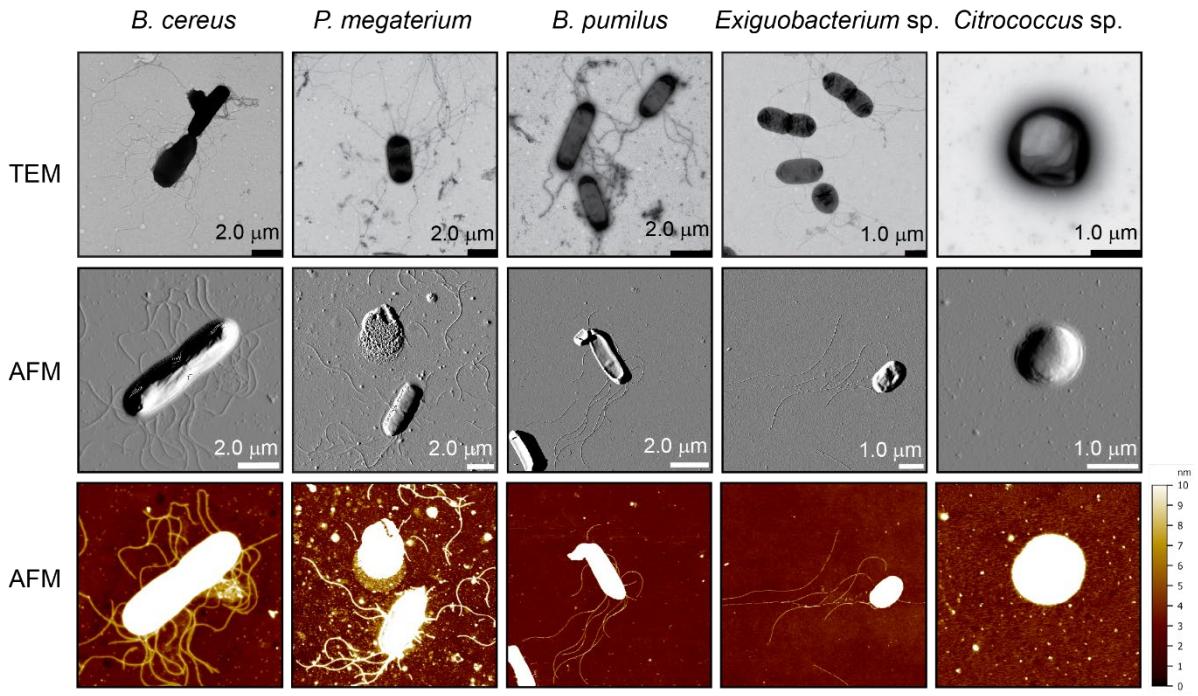


Fig. S11. High resolution imaging of bacterial species. Transmission electron microscopy **(**TEM) and atomic force microscopy (AFM) images of the indicated bacterial species. The bottom panels show the same AFM images as in the middle panels, with highlighted flagella in height mode. The height color scheme on the right is valid for all the species. The images are representative of two independent experiments.

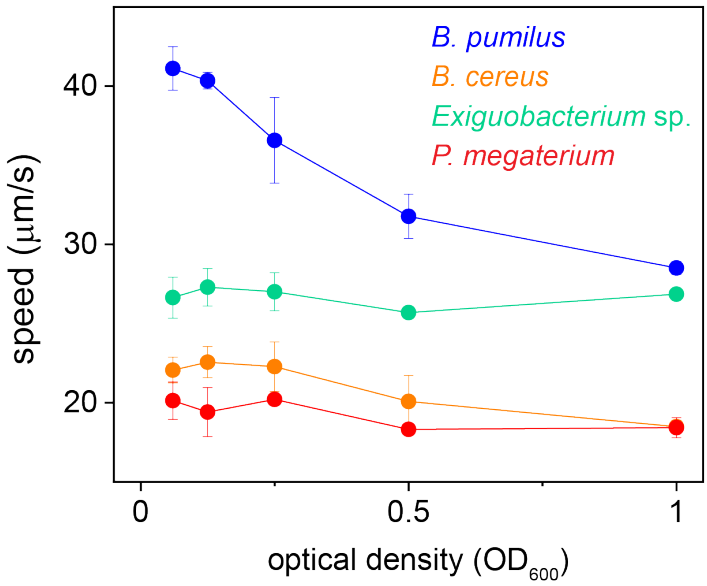


Fig. S12. Bacterial speed as a function of bacterial concentration under bioconvective conditions. Mean speed as a function of bacterial concentration for the indicated bacterial species (*B. pumilus*, blue; *P. megaterium,* red; *B. cereus,* orange and *Exiguobacterium* sp., green), obtained by dilution of initial samples characterized by OD_{600}=1 (see Methods). The values of the speeds were obtained by averaging over all trajectories from at least two independent experiments, with at least five technical repeats in each, and standard error bars were estimated from 10000 bootstrap samples. The speeds were computed from the same traces used to compute persistence lengths in Fig. 5b. The number of trajectories n for each value of OD_{600} and species is given in the table below for different experimental repeats (Exp).

***Exiguobacterium* sp**

OD_{600}	Exp1	Exp2	**Total**
1.0	5574	4216	9790
0.5	4728	3412	8140
0.25	2399	2056	4455
0.12	1644	1159	2803
0.06	935	609	1544

P. megaterium

OD_{600}	Exp1	Exp2	Exp3	**Total**
1.0	3632	3243	3994	10869
0.5	2740	918	2633	6291
0.25	839	1081	721	2641
0.12	911	353	1039	2303
0.06	223	435	605	1263

B. cereus

OD_{600}	Exp1	Exp2	**Total**
1.0	1707	2341	4048
0.5	863	1433	2296
0.25	347	796	1143
0.12	200	843	1043
0.06	95	419	514

B. pumilus

OD_{600}	Exp1	Exp2	**Total**
1.0	6345	6400	12745
0.5	7302	8477	15779
0.25	3529	7822	11351
0.12	2191	2738	4929
0.06	2367	5345	7712

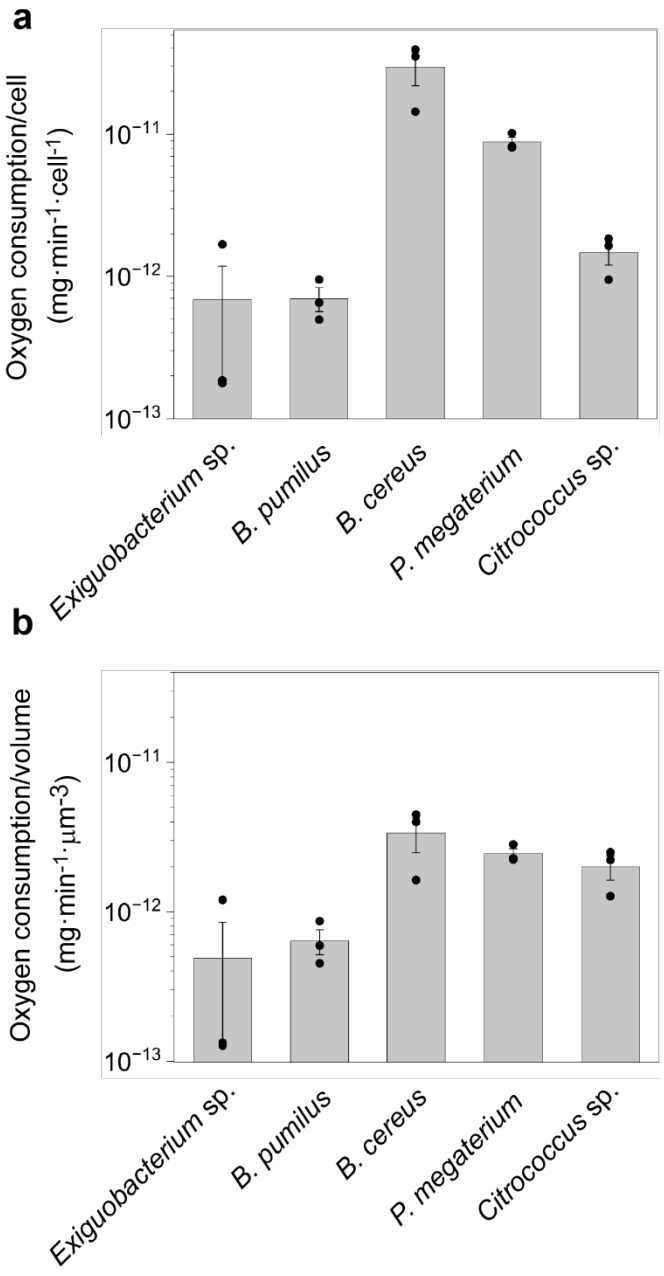


Fig. S13. Oxygen consumption rate for the indicated species. a Mean oxygen consumption rate per cell (see Methods). **b** Mean oxygen consumption rate normalized by the average cell volume. Error bars for a and b denote standard errors of the mean calculated from three independent experiments. Average cell volumes calculated from measurements of cell length and width from at least 50 cells were: 1.4±0.2 μm^3 (*Exiguobacterium* sp.), 3.6±0.3 μm^3 (*P. megaterium*), 1.1±0.3 μm^3 (*B. pumilus*), 8.8±0.4 μm^3 (*B. cereus*) and 0.7±0.2 μm^3 (*Citrococcus* sp.*)*.

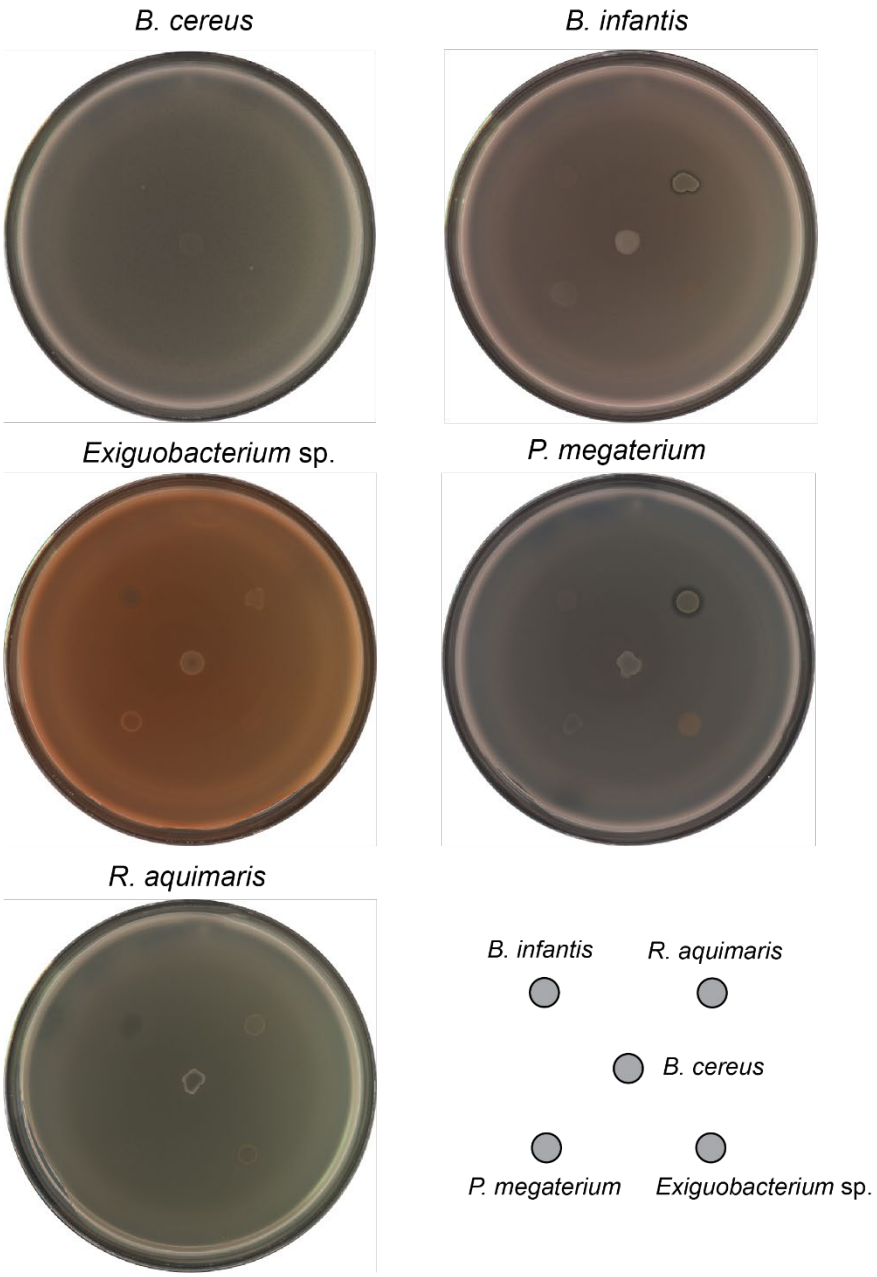


Fig. S14. Pairwise interactions as measured by spot-lawn assays. Spot-lawn assays were performed to determine the antagonism interactions among the species used in five-species bioconvection experiments (Fig. 6). The species with which a lawn was sown is given at the top of each dish and five species were added as a spot according to the given scheme. The images are representative of three independent experiments.

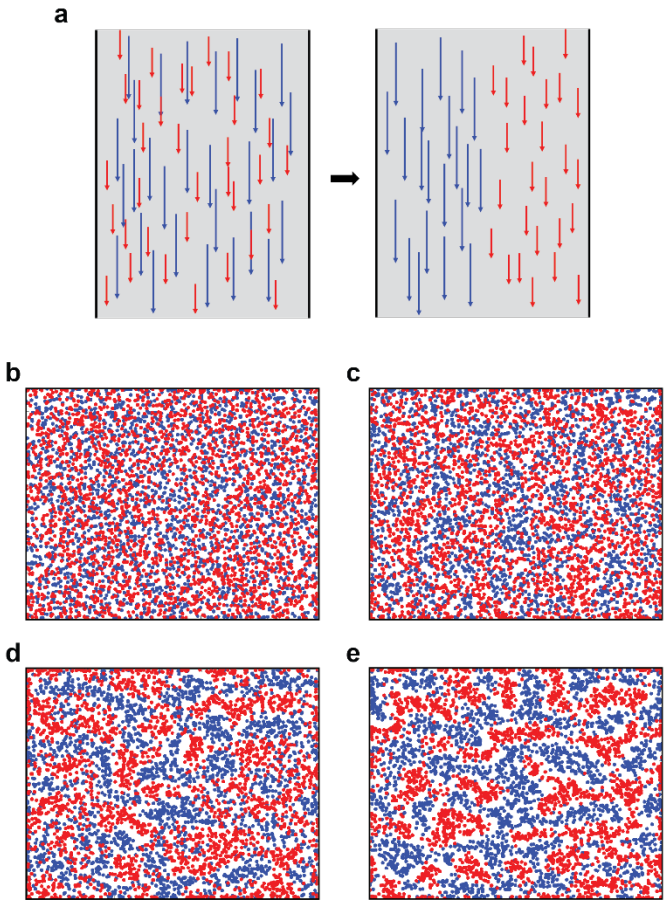


Fig. S15. Simulation of a toy model of a three-dimensional binary mixture of particles with different speeds, moving in the same direction. a Schematic depiction of segregation viewed from the side in a toy model of a mixture of two particle types (red and blue) moving in the same direction (down), with different speeds represented by the arrow length, from a mixed state in which there are collisions (left) to a segregated state (right), in which there are no collisions between like particles. Collisions result in lateral motion that promotes segregation. Particles reaching the bottom are re-injected from the top (periodic boundary conditions). **b-e** Typical top view snapshots from simulations in three dimensions of segregation in binary mixtures of particles with different speeds contrasts (blue particles have a fast speed $\boldsymbol{u_f}$ and red particles have a slow speed $\boldsymbol{u_s}$): $\boldsymbol{u_s} = \mathbf{1}$ and $\boldsymbol{u_f} = \mathbf{1}$, 1.1, 1.25 and 1.5 respectively. In all cases the snapshots were recorded at the same time after the onset from a well-mixed state (see deposited code). Note that orientational noise was included in all the simulations.

Table S1. List of strains used in this study. Species and strains deposited and registered in the World Data Centre for Microorganisms, under the acronym CDBB-500 (https://ccinfo.wdcm.org/details?regnum=500).

Strain	Species	Registration number in CDBB	Reference
GOB958	*Exiguobacterium* sp.	CDBB-B-2122	
CH_111	*Bacillus cereus*	CDBB-B-2119	[5]
CH_111 *[pNW-sfGFP(SPS6)]*	*Bacillus cereus*	CDBB-B-2123	
CH_447	*Priestia megaterium*	CDBB-B-2121	[5]
CH_145	*Bacillus pumilus*	CDBB-B-2120	[5]
CH_26a	*Citrococcus sp.*	CDBB-B-2116	[5]
CH_87b	*Rossellomorea aquimaris*	CDBB-B-2118	[5]
CH_37	*Bacillus infantis*	CDBB-B-2117	[5]

References

1. Schindelin, J. *et al.* Fiji: an open-source platform for biological-image analysis. *Nat Methods* **9**, 676–682 (2012).
2. Amsler, C. D., Cho, M. & Matsumura, P. Multiple factors underlying the maximum motility of Escherichia coli as cultures enter post-exponential growth. *J. Bacteriol.* **175**, 6238–6244 (1993).
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4. Aguilar-Salinas, B. & Olmedo-Álvarez, G. A three-species synthetic community model whose rapid response to antagonism allows the study of higher-order dynamics and emergent properties in minutes. *Front. Microbiol.* **14**, 1057883 (2023).
5. Pérez-Gutiérrez, R. *et al.* Antagonism influences assembly of a Bacillus guild in a local community and is depicted as a food-chain network. *ISME J.* **7**, 487–497 (2013).
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